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APPLICATION AND DEVELOPMENT REPORT
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THE USE OF PHLOXINE B AND ROSE BENGAL STAINS TO FACILITATE SORTING BENTHOS SAMPLES

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PREFACE

The use of stains to facilitate sorting benthos samples was developed in response to a need for a more accurate and time-saving method of separating organisms from the detritus. The study was initiated in February 1966 by William T. Mason, Jr., an aquatic biologist, under the direction of Mr. Joseph B. Anderson who serves as Chief, Aquatic Biology for this Division. Paul Yevich is a histopathologist, formerly with the Division of Occupational Health, U.S. Public Health Service, Cincinnati. Mr. Yevich is now with the National Marine Water Quality Laboratory, Kingston, Rhode Island.

Valuable advice was given by Dr. Cornelius I. Weber of this Division. Helpful information was provided in correspondence with Dr. Charles R. Goldman, Professor of Zoology, University of California at Davis.

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THE USE OF PHLOXINE B AND ROSE BENGAL STAINS TO FACILITATE SORTING BENTHOS SAMPLES

One of the most tedious and time-consuming tasks in gathering benthos data is sorting the organisms from the debris. To conserve time, benthic investigators have employed mechanical devices. The sugar flotation method (Anderson, 1959) has been used in studies dealing with sandy substrates. This method will not yield consistent results if the samples contain more than slight quantities of organic detritus. Whitehouse and Lewis (1966) utilized carbon tetrachloride to separate the organisms and detritus from samples composed primarily of sand and gravel by flotation. This method was not tested for samples containing other substrates and there are obvious safety hazards with continued use of carbon tetrachloride.

The addition of rose bengal to benthic samples preserved in formalin was mentioned in a comprehensive water resources study of Lake Tahoe, Nevada, conducted by biologists from the University of California at Davis and Engineering Sciences, Incorporated (1963). However, the concentrations used and results obtained in using the stain are not reported. Hand sorting samples with the aid of a low power scanning lens is still the most widely used method of separation (Standard Methods, 1965).

The xanthene stains, phloxine B ($C_{20}H_2O_5Cl_4Br_4Na_2$:molecular weight 829.702) and rose bengal ($C_{20}H_2O_5Cl_4I_4K_2$:molecular weight 1049.884), have been used by microbiologists to stain animal tissues and microorganisms (Gurr, 1960). They are relatively inexpensive ($11.25/100 gms. and $17.40/100 gms., respectively) and can be obtained through chemical and biological supply houses.

METHODS

Ekman dredge samples collected from different substrate types in the Klamath and Lost Rivers, Oregon, were used in a time test to determine if staining reduces sorting time. Three dredge series, each composed of six samples, were collected in close proximity from substrates composed predominantly of sand-silt or organic detritus. The samples were washed in a U. S. No. 30 sieve and preserved in either 70% ethanol or 5% formalin.

In the laboratory three of the samples in each series were stained with phloxine B, at a concentration of 100 mg/l, by diluting the sample preservative with a stain-preservative stock solution (200 mg/l stain in 95% ethyl alcohol or 10% formalin). The remaining three samples were used as controls. The jars were agitated once to ensure that all the animals would have contact with the stain.

After two days the samples were rinsed in a U. S. No. 40 sieve to remove excess stain. Aliquots (100 cc) of the samples were

transferred from a beaker to a shallow glass Petri dish and examined for organisms under a 2X scanning lens, utilizing direct light on a white background. Recovered organisms were placed in vials containing 70% ethanol and later enumerated and identified. All samples were sorted by the same individual using identical techniques throughout, and a record was made of the sorting time.

DISCUSSION AND RESULTS

Benthos samples stained with phloxine B at a concentration of 100 mg/l in ethanol and formalin preservatives were sorted in approximately half the time required for unstained samples (Table I). Nine phloxine B stained samples containing 2748 organisms were sorted in 950 minutes (an average of 2.9 organisms per minute), whereas the nine unstained samples with 2225 organisms were sorted in 1725 minutes (an average of 1.3 organisms per minute). The time saved in sorting benthos samples will of course depend on the amount of detritus, number of organisms, and the skill of the sorter. The stain concentration must be adjusted to suit the particular substrate type and increased if the samples are composed primarily of organic detritus.

The organisms in the stained benthos samples became bright red after 48 hours which offered good visual contrast to the detritus.

They stained equally well in samples composed primarily of sand, silt, or organic detritus. Many of the smaller macroinvertebrates, easily overlooked, were readily apparent in the stained samples.

Rose bengal has been used in samples at a concentration of 100 mg/l with equal effectiveness in reducing the sorting time. It has advantage over phloxine B in that good staining results in 24 hours and the red color is more vivid.

A stain-preservative stock solution of ethanol or formalin can be used in the field to stain the samples thus eliminating excess handling in the laboratory. In turn the organisms will have a longer period of contact with the stain before sorting.

Variations in coloration are often important taxonomic characteristics in keying aquatic macroinvertebrates. If necessary, most of the stain can be removed from the organisms by placing them in 95% ethyl alcohol. As yet the stains have not interferred with specific determinations based on color distinctions.

Table I. Minutes Required to Sort Ekman Dredge Benthos Samples Stained with Phloxine B

Sample	Organic Detritus in 70% EtOH				Sand-Silt in 70% EtOH				Organic Detritus in 5% formalin			
	Stain		Control		Stain		Control		Stain		Control	
	Minutes	Animals Sorted	Minutes	Animals Sorted	Minutes	Animals Sorted	Minutes	Animals Sorted	Minutes	Animals Sorted	Minutes	Animals Sorted
1	208	1176	280	276	140	340	272	748	60	140	116	148
2	120	236	140	112	144	172	156	204	56	232	108	116
3	148	304	480	128	22	36	140	444	52	112	33	49
Total	476	1716	900	516	306	548	568	1396	168	484	257	313

Total time, stained samples = 950 min:Number individuals sorted = 2748

Total time, unstained samples = 1725 min:Number individuals sorted = 2225

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